ORIGINAL ARTICLE

Comparison of Immunohistochemical and Fluorescence *In Situ* Hybridization Assessment for HER-2/neu Status in Taiwanese Breast Cancer Patients

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SUMMARY

Objective: Accurate diagnostic assessment of human epidermal growth factor receptor-2 (HER-2) is essential and a prerequisite for appropriate application of the humanized anti-HER-2 monoclonal antibody trastuzumab (Herceptin) to the treatment of patients with breast cancer. Immunohistochemistry (IHC) is the most widely applicable diagnostic modality in studying HER-2 status. Fluorescence *in situ* hybridization (FISH) is also recognized as a modality in cases with an equivocal IHC status (score, 2+). Some authors claimed that FISH alone is sufficient. The aim of this study was to correlate the test results of IHC and FISH for *HER-2* gene amplification in breast cancer patients. FISH for topoisomerase IIα (TOP2A) was also studied to see if deletion or amplification of TOP2A has any supplementary role to HER-2, FISH and IHC.

Materials and Methods: Assessment of *HER-2* gene amplification and *TOP2A* gene amplification/deletion was made by FISH analysis using the LSI TOP2A/HER-2/CEP 17 multicolor probe or the LSI HER-2/CEP dual color probe (Vysis, Downers Grove, IL, USA) in formalin-fixed and paraffin-embedded tissue sections of 54 breast cancer patients who were grouped into stages 1+, 2+ or 3+ based on IHC (HercepTest; DakoCytomation, Carpinteria, CA, USA) observations.

Results: None of IHC 1+ breast tumors was HER-2 FISH positive, but three of 18 (17%) IHC 3+ tumors were HER-2 FISH negative. Overall, 53% of the IHC 2+ and 83% of the IHC 3+ cases were HER-2 FISH positive. Only one case with IHC 3+ tumor that was HER-2 FISH positive was found to have TOP2A amplification (> 2.0) and no IHC 2+ cases were found to have TOP2A amplification. There were no cases with TOP2A deletion (< 0.8) in our whole series. There were also no cases of HER-2 FISH negative tumors, but IHC scored as 2+ or 3+ (0 of 10), to be found with TOP2A amplification. The discordance rates by IHC were high (46.7% in IHC 2+, 16.7% in IHC 3+, 30.3% overall in IHC 2+ or 3+). On the contrary, the discordance rates were zero if by FISH.

Conclusion: The current algorithm to use HER-2 FISH as a supplementary role to IHC HercepTest 2+ may need some modifications according to the local setting. TOP2A FISH adds little value to HER-2 FISH and IHC staining in our study. [*Taiwan J Obstet Gynecol* 2007;46(2):146–151]

Key Words: breast cancer, fluorescence *in situ* hybridization, HER-2, immunohistochemistry, topoisomerase IIα

Introduction

The human epidermal growth factor receptor-2/neu gene (*HER-2/neu*) is a proto-oncogene that is overamplified and/or overexpressed in up to 30% of patients with

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breast cancer [1]. The overexpression of *HER-2/neu* is associated with increased tumor growth rate, enhanced metastatic rate, shorter disease-free survival, and overall survival. It is well-known that patients with *HER-2/neu* (formerly known as *c-erbB-2*) overexpressing tumors have a more aggressive and more malignant course than patients without such overexpression, as seen in Taiwanese breast cancer patients [2], a finding similar to studies reported elsewhere [3].

Overexpression of *HER-2/neu* can be studied at the levels of DNA, mRNA and protein, using fluorescence *in situ* hybridization (FISH), Northern blot, and Western blot and immunohistochemistry (IHC), respectively. Amongst them, IHC and FISH are the two most accessible and feasible methodologies used in clinical diagnosis.

FISH analysis has enhanced the sensitivity of the standard karyotype and permitted the detection of submicroscopic abnormalities. With this new method, targeted DNA sequences are visualized, permitting analysis and quantification of disease at the molecular level. It is more sensitive than standard cytogenetics since it can survey many more cells at interphase. In the process of FISH analysis, the probe is labeled with a fluorescent dye. The probe binds to the chromosome *in situ*, and the location of the hybridized fragment is revealed by a bright fluorescent signal. FISH permits analysis of both cell cultured materials and paraffin-embedded archival tissues, the latter being particularly useful when used in a clinical diagnostic setting.

HER-2/neu has been targeted by a variety of strategies, including monoclonal antibodies, immunoconjugates and vaccines. A targeted immunotherapy, using a humanized monoclonal antibody, trastuzumab (Herceptin; Genentech, South San Francisco, CA, USA), was devised to tackle breast carcinomas with overexpression of *HER-2/neu*. The US Food and Drug Administration (FDA) approved an immunohistochemical assay (HercepTest; DakoCytomation, Carpinteria, CA, USA) to assess the HER-2 status in formalin-fixed, paraffin-embedded tissue of breast carcinoma in 1998. However, reports showed a false-positive rate ranging from 6% to more than 50% with the HercepTest. Therefore, FISH test for HER-2 status was proposed [4,5]. However, despite the fact that most studies revealed high concordance rates of HER-2 FISH when IHC is 0/1+ or 3+, a significant discordance was observed between HER-2 FISH and IHC 2+ and between IHC 2+ and mRNA data. Some authors even claimed to find a very high discordance rate between HER-2 FISH and IHC in all four IHC scores (0, 1+, 2+, 3+), and a FISH-alone screening strategy was alternatively suggested [6]. Meanwhile, a strategy to screen HER-2 status was developed by some laboratories, and the strategy was that IHC be used first to screen the specimens of breast cancer. Expression of HER-2/neu protein was then scored as 0, 1+, 2+ or 3+. Herceptin was prescribed to patients whose IHC for *HER-2/neu* was 3+. Those with a score of 2+ received an adjunct FISH analysis by using a probe aimed at *HER-2/neu* (17q11.2–12) to see if there was an amplification of the *HER-2/neu* proto-oncogene. Herceptin was given only in those if HER-2 FISH was positive for amplification, even though the IHC score was only 2+.

There is still some controversy in the discordance rate between IHC and FISH. Some studies revealed a high discordance rate between FISH and IHC in all four IHC scores (e.g. 92% discordance rate in the study by Dolan and Snover [4]) [5], which contradicted with the conclusion held by many investigators that a high discordance rate might only exist in the IHC 2+ group [7]. Factors, including the experience of the pathologists who performed the IHC analysis, the standard of the cytogenetic laboratory, the method of obtaining specimens (fine needle aspiration, biopsy, or surgical specimen) and the composition of the patient population, may all influence the interpretation of IHC and FISH analysis. We wanted to perform our own analysis in our own local setting to see the concordance and discordance rates between the two tests (IHC and HER-2 FISH). Besides, topoisomerase IIα (TOP2A) was noted to co-amplify or was deleted in tumor tissue with HER-2 amplification. We were also interested in seeing if TOP2A FISH was helpful in amending the discordance of the two more widely applied methodologies: IHC and HER-2 FISH, and thereby reducing the risk and the cost of unnecessary exposure to Herceptin. A few other groups from Taiwan have studied the related issues; one previous study reported that overexpression of *HER-2/neu* correlated well to tumor staging, survival, and lymph node metastasis, which was similar to the reports from other parts of the world, apart from the finding that the percentage of *HER-2/neu* overexpression was higher in Taiwanese than in Caucasians [2]. Other groups reported that overexpression of HER-2/neu protein is common in both familial and non-familial breast cancers and correlated well to the metastasis-free survival [8–10]. A similar study of a larger scale that was conducted at the National Health Research Institute of Taiwan (NHRI) targeted at studying the correlation between HER-2 FISH and IHC 2+ and recommended a similar strategy of HER-2 screening in breast cancer patients, i.e. to use IHC as a primary tool and HER-2 FISH as an adjunct when the IHC score is 2+ [11]. We accumulated experience, during 2002 to 2005, as a single institute by close collaboration with the cytogenetic laboratory and the pathology department (both are one of the largest

laboratories of its kind in central Taiwan) and were keen to see whether our experience follows the trend reported previously by other groups in Taiwan.

Materials and Methods

Samples

A total of 54 formalin-fixed, paraffin-embedded tissue sections were retrieved from the tissue bank of our pathology department. The record revealed that the specimens belonged to 54 different patients with primary breast cancer of different stages during 2003 to 2005 who received excisional biopsy. The samples were then sent to the pathologists and the cytogeneticists separately, with both groups not knowing each other's results until the data were incorporated together for analysis.

Immunohistochemical analysis

Immunohistochemical study was performed on paraffin-embedded, formalin-fixed tissue sections using the HercepTest (DakoCytomation, Carpinteria, CA, USA) following the manufacturer's instructions. Briefly, this procedure included the deparaffinization and rehydration steps, followed by an epitope retrieval step in which the tissue sample was incubated in a citrate buffer solution at 90–95°C for 20 minutes. The slide was then subjected to a series of alternating washes in tris(hydroxymethyl)aminomethane hydrochloride buffer and incubation steps with, first, a peroxidase-blocking reagent for 5 minutes and then with HER-2 primary antibody, followed by a visualization reagent (dextran polymer conjugated with horseradish peroxidase and goat anti-rabbit immunoglobulins) for 30 minutes each, and finally with a 3,3′-diaminobenzidine chromogen solution. After a final wash, the slide was counterstained with hematoxylin [4].

Scoring was performed according to the manufacturer's recommendations by pathologists with at least 5 years of experience in clinical practice and was finally reviewed by one of the coauthors (Yeh) who had particular experience in breast pathology and immunohistochemical interpretation of HER-2 testing.

FISH analysis

HER-2 FISH was performed on formalin-fixed, paraffin-embedded tissue specimens from each of the 54 patients using the PathVysion kit (Vysis, Downers Grove, IL, USA). Included in this kit are probes to the *HER-2* gene locus at 17q11.2–12 (labeled with SpectrumOrange) and to the centromeric region of chromosome 17 (CEP 17; labeled with SpectrumGreen). TOP2A FISH was performed on formalin-fixed, paraffin-embedded tissue specimens from each of the 54 patients using the locus specific identifier (LSI) TOP2A kit (Vysis, Downers Grove, IL, USA). Included in this kit are probes to the *TOP2A* gene locus at 17q21–22 that encodes topoisomerase II (labeled with SpectrumOrange) and to the centromeric region of chromosome 17 (CEP 17; labeled with SpectrumGreen). FISH analysis was performed according to the manufacturer's instructions (after obtaining proficiency certification on completion of training by Vysis), with minor modifications as described subsequently. Our laboratory passed the accreditation of cytogenetics and FISH testing by the College of American Pathologists and by the supervising body in our government (Department of Health, Taiwan).

Briefly, unstained 3- to 5-μm thick paraffin sections were cut from blocks chosen by the referring pathologists and placed on positively charged slides. On receipt, the slides were placed in an oven at 94°C for approximately 5 hours, deparaffinized in xylene, and dehydrated in a series of ethanol washes. After pretreatment in 0.2 N hydrochloric acid and sodium thiocyanate solutions, digestion in a protease solution for 16 minutes, and fixation in 10% neutral buffered formalin, the slides were subjected to denaturation and hybridization with 10 μL of the PathVysion probe/buffer mixture.

Fluorescence microscopy integrated with a cooled CCD camera system and Smart Capture software (CytoVision Chromophour System; Applied Imaging Ltd., Carlsbad, CA, USA) for chromosome arrangement was used to investigate and analyze the FISH results.

Scoring was performed according to the manufacturer's recommendations by cytogenetic technologists with at least 2 years of experience in clinical testing and was finally reviewed by two of the coauthors (Chen and Wang) who both had particular experience in molecular cytogenetics.

Results

Of the 54 specimens in our study (25, invasive lobular carcinoma; 29, invasive ductal carcinoma with varying tumor grades and clinical stages), none was classified as IHC 0, 21 were classified as IHC 1+, 15 were classified as IHC 2+, and 18 were classified as IHC 3+ by HercepTest. None of the 21 IHC 1+ cases was found to be HER-2 FISH positive (amplified *Her-2/neu* with a *Her-2/neu* to CEP 17 ratio > 2.0 by counting at least 100 interphase nuclei; Figure 1), eight of the 15 IHC 2+ cases and 15 of the 18 IHC 3+ case were found to be HER-2 FISH positive (Table 1). No statistical significance between the tumor cell type and IHC status was noted (data not shown).

Discordance was defined as a discrepancy existing between the IHC and HER-2 FISH, including the following two conditions: (1) IHC 2+ or 3+ but HER-2 FISH negative (a *Her-2/neu* to CEP 17 ratio < 2.0 by counting at least 100 interphase nuclei); (2) IHC 1+ but HER-2 FISH positive. The discordance rate according to IHC 2+ was defined as the number of discrepant IHC 2+ cases divided by the total number of IHC 2+ cases and was 46.7% (7/15). The discordance rate according to IHC 3+ was defined as the number of discrepant IHC 3+ cases divided by the total number of IHC 3+ cases and was 16.7% (3/18). The overall discordance rate by IHC was therefore 30.3% (10/33). Following the same way of counting, we easily found that the discordance rates by FISH were 0 and 0 (Table 2).

Only one case with IHC 3+ and HER-2 FISH positive was found to have an amplified *topoisomerase IIα* (TOP2A FISH positive). All the other 53 cases were found to have a normal copy number of *TOP2A* (ratio, 0.8–2.0) with very good quality signals (Figure 2). Remarkably, there was no failed hybridization in the FISH analyses we performed in this study.

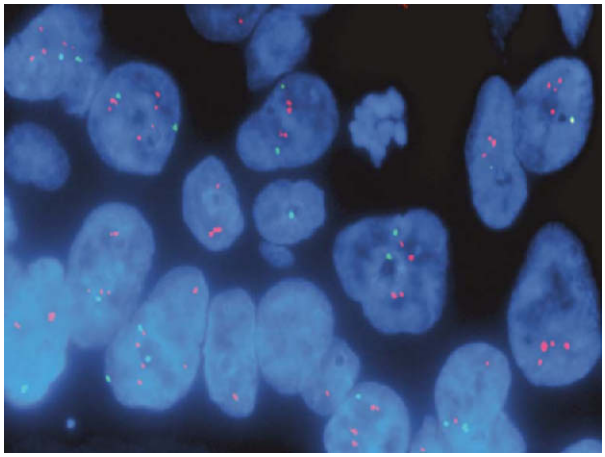

Figure 1. Marked amplification of human epidermal growth factor receptor-2/neu (Her-2/neu) gene locus was noted. The ratio of Her-2/neu (red signals) to CEP 17 (green signals) is obviously larger than 2.0.

Discussion

Since the research regarding the role of *Her-2/neu* in breast carcinogenesis led to the development of the targeted immunotherapeutic monoclonal antibody trastuzumab (Herceptin), diagnostic laboratory tests are being investigated thoroughly to help detect the overexpression of *Her-2/neu* in patients with breast cancer with acceptable sensitivity and specificity suitable for clinical use. The biological events underlying HER-2-driven breast cancer that can be assessed in routine clinical specimens include evaluation of gene amplification by FISH, enhanced messenger RNA expression by reverse transcription polymerase chain reaction and Northern blotting, and the assessment of protein overexpression at the tumor cell membrane by IHC. The US

Table 1. Results of HercepTest—immunohistochemistry (IHC), human epidermal growth factor receptor-2 (HER-2)—fluorescence *in situ* hybridization (FISH), and topoisomerase IIα (TOP2A)—FISH

IHC scoring	Number of patients	Distribution of HER-2 FISH amplified (> 2.0 and denotes amplified HER-2/neu gene)	Distribution of TOP2A FISH amplified (> 2.0 and denotes amplified TOP2A gene)	Distribution of TOP2A-FISH deleted (< 0.8 and denotes deleted TOP2A gene)
1+	21	0	0	0
2+	15	8	0	0
3+	18	15	1	0

Table 2. Concordance between HercepTest and human epidermal growth factor receptor-2 (HER-2)—fluorescence *in situ* hybridization (FISH) results

HercepTest	HER-2 FISH amplified	HER-2 FISH non-amplified	Concordance by IHC score (%)	Discordance by IHC score (%)
1+ (n = 21)	0	21	100	0
2+ (n = 15)	8	7	53.3	46.7
3+ (n = 18)	15	3	83.3	16.7
Concordance by FISH score (%)	100	100	—	—
Discordance by FISH score (%)	0	0	—	—

IHC = immunohistochemistry.

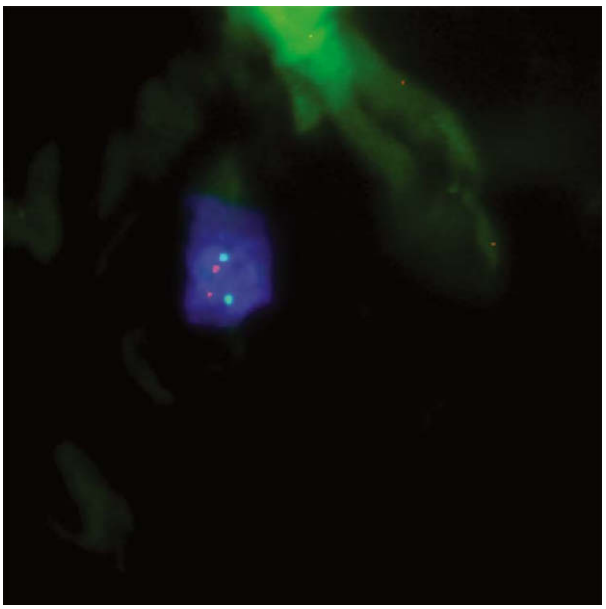

Figure 2. Topoisomerase IIα (TOP2A) fluorescence *in situ* hybridization (FISH) revealed a normal pattern of topoisomerase II (red signals) when compared with the internal control aimed at centromere 17 (green signals).

FDA approved the immunohistochemical test HercepTest in 1998 and the FISH test Her-2 FISH pharmDx™ Kit (DakoCytomation, Carpinteria, CA, USA) in 2005 to mark the fact that IHC study to detect Her-2/neu protein overexpression and FISH to detect an increase in gene copy number of Her-2/neu locus are the two mainstream diagnostic tests of choice in the daily clinical practice.

We did not intend to compare the sensitivity and specificity of these two tests, since a great number of extensive studies had been published [12–14]. Besides, the case number in our study ($n = 54$) was also not that large for us to draw any meaningful statistical correlation between the two tests and the clinical parameters such as tumor staging, grading, cell types, the status of nodal involvement, and patient survivals. Many previous studies in Taiwan had demonstrated the role of *Her-2/neu* overexpression in breast carcinogenesis in Taiwanese, and the data were comparable to those reported elsewhere in the world [2,8–10]. This study wishes to answer a simple question: what are the concordance and discordance rates between IHC and HER-2 FISH in our own setting, and is TOP2A FISH helpful to the discordant cases? A similar study reported by Dolan and Snover [4] adopted a similar strategy, but they represented a report from a referral laboratory that receives samples referred from many surgical departments, which is different from us and the samples in their study might be of a more heterogeneous nature than those in our study. Another study from our NHRI also represented a core FISH laboratory that receives samples referred from a number of hospitals across the country [11]. Many factors such as a greater variability in the quality of sample preparation from many pathology departments and a greater interpersonal variability, thus, may cloud the result of those studies, despite that the conditions in those two studies actually also reflect part of the true condition encountered in the daily clinical practice. Our study, therefore, wishes to answer the question in a much simpler and less clouded setting and also reflects our own real condition in clinical routine practice. Considerably high discordance rates in our study (46.7% in IHC 2+ and 16.7% in IHC 3+) and in a previous study from the US (86% in IHC 2+ and 54% in IHC 3+) imply the inherent differences in sensitivity and specificity of these two testing methods. Moreover, the quality of sample preparation also affects the performance of these two tests. Result of FISH analysis is easily jeopardized by poor preparation (too thickly sectioned, unsatisfactory deparaffination, tissue damage). However, poor quality is more dangerous in IHC results, because it will lead to a wrong assignment of the samples to "negative" result [15]. Fortunately, we did not have any IHC 0 cases included in our study and the influence of such a factor would be lessened. A French multi-centered study, using FISH as a gold standard, also revealed that IHC scoring in HER-2-status needs a more standardized cut-off level (e.g. more than 60% stained cells were classified as overexpression) to improve its concordance with FISH, implying the fact that IHC scoring is somewhat subjective and prone to interpersonal variation [16].

Theoretically, to use a FISH-alone strategy in breast cancer patients with *Her-2/neu* overexpression may miss those who have overexpression of Her-2/neu at protein level but do not have copy number change at DNA level, the prevalence of which is approximately 3% [12]. Whether we can change our algorithm to FISH alone, since now our data revealed the discordance by FISH is zero, awaits further study; while at least in our hospital with a sound FISH laboratory, change in algorithm may be a reasonable alternative and must be examined by correlating with the clinical parameters in the future.

Meanwhile, in order to solve the discrepancy between the HER-2 FISH and HercepTest IHC, the role of *topoisomerase IIα* was studied. The study from our NHRI reported that the *topoisomerase IIα* gene was co-amplified in 21% and was deleted in 15% of HER-2/neu amplified samples, which implies that amplification or deletion of *topoisomerase II* gene may have some supplementary value in the diagnosis of breast cancers with overexpression of *HER-2/neu*. On the contrary, a study coauthored by Hicks et al [17] indicated that TOP2A was

never amplified in the absence of HER-2 amplification and was co-amplified in 50% of HER-2 amplification cases; HER-2 gene copy number was higher than the TOP2A copy number ($p < 0.01$). However, in our study series, TOP2A was only co-amplified in 6% of IHC3+ cases. We also did not observe any deletion cases in TOP2A FISH. Our study revealed that TOP2A FISH has little value to be an adjunct test to the current algorithm. We, therefore, have aborted the TOP2A FISH for such use in our hospital since 2006.

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